5-2012

Sweetgum Bark: Extraction, Purification, and Determination of Antioxidant Activity

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4/5/2012

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Acknowledgements

I would like to thank Dr. Danielle Julie Carrier for allowing me to work in her lab. This experience has taught be a lot about the growing topic of cellulosic biofuels and encouraged me to become a better engineer. I would also like to thank Dr. Betty Martin, Ching-Shuan Lau, Kris Bunnell, and Angele Djioleu for all of the guidance they have given me throughout the last two years; especially with regards to operating and fixing the HPLC and CPC. Additionally, I would like to thank Dr. Carl Griffis and Dr. Tom Costello for serving as my committee members for my defense. Finally, I would like to thank the Arkansas Science and Technology Authority for awarding me the NSF EPSCoR Student Undergraduate Research Fellowship during the spring and summer of 2011.

Abstract

Sweetgum (*Liquidambar styraciflua* L.) is a native tree species that grows as a volunteer in the understory of managed pine forests in Arkansas. Sweetgum, as well as other volunteer species, must be removed before the pines can be harvested. Sweetgum wood can be converted to hemicellulosic and cellulosic sugars by dilute acid pretreatment and enzymatic hydrolysis. These wood-derived sugars can then be fermented into cellulosic ethanol. Usually, the bark is not deconstructed into sugars, but transformed into thermal energy via a combustion process. Interestingly, the bark of the sweetgum contains value-added products that could be recovered prior to thermal processing. Recovering value-added compounds will increase the tree's value and potential to be used as a biofuel-destined feedstock.

The goal of this research was to extract value-added chemicals from sweetgum bark, and to determine their antioxidant capabilities. The specific compounds contributing to antioxidant activity were identified and separated using high pressure liquid chromatography (HPLC) and centrifugal partition chromatography (CPC). The amount of antioxidant activity in the bark and the individual fractions was determined using the thiobarbituric reactive substance (TBARS) assay.

Introduction

With the use of energy being greater than ever and fossil fuels being rapidly depleted, it is necessary to design renewable energy systems that are competitive both economically and socially with the existing fossil fuel energy system. It is imperative that the renewable energy system of the future combine the use of solar, wind, tidal, biomass, and fossil fuels energy. In terms of biomass, the conversion of a non-food biomass into biofuels for energy is becoming increasingly popular. Current choices of cellulosic biomass include, among others, switch grass, poplar, algae, and sweetgum. Sweetgum trees (*Liquidambar styraciflua* L.) are a good choice because they grow rapidly and abundantly, and are currently part of the existing forestry supply-chain. Sweetgum is a native hardwood species that grows as volunteers in the understory of managed pine forests in much of the southeastern United States [1]. Sweetgum, as well as other volunteer species, must be removed before the pines can be harvested.

Sweetgum can be converted to hemicellulosic and cellulosic sugars by dilute acid pretreatment followed by enzymatic hydrolysis. These sugars can then be fermented into cellulosic ethanol. This can easily be done with sweetgum wood. However, the bark is more recalcitrant and is not easily deconstructed into its monomeric sugar components. However, sweetgum bark contains value-added compounds that may be recovered and incorporated into everyday products. This would increase the economic value of the feedstock and its potential to be used as a biofuel-destined biomass.

In folkloric literature, sweetgum is documented to be used as an antibiotic and pain reliever. Recently sweetgum bark was determined to contain the value-added compound shikimic acid as described in Martin et al. [1]. Shikimic acid has been used historically for medical

purposes [1]. It is currently used in the anti-influenza drug Tamiflu®. The main sources of shikimic acid are the Chinese star anise and fermentation from *Escherichia coli* [1], however the compound is still in very high demand and sweetgum could prove to be a potential source of shikimic acid as well as a potential biofuel-destined feedstock [1].

It is likely that sweetgum bark also contains other valuable phytochemicals which would further increase the value of sweetgum. The purpose of this research is to extract the value-added chemicals that can be removed from the biomass prior to conversion into biofuels, and to determine their antioxidant capabilities. Antioxidants are important for many reasons. Specifically, antioxidants inhibit the oxidation of low density lipoproteins (LDL). Oxidation of LDL is detrimental to one's health and causes disease such as atherosclerosis [2]. Potential compounds in the bark contributing to the antioxidant activity will be identified using high pressure liquid chromatography (HPLC). The compounds will then be separated using centrifugal partition chromatography (CPC). The antioxidant activity in the bark, fractions, and the standard compounds will be determined using the thiobarbituric acid reactive substances (TBARS) assay.

Materials and Methods

Biomass:

Sweetgum bark chips from mature trees were obtained from Dr. Matthew Pelkki and Dr. Philip Tappe, School of Forest Resources, University of Arkansas, Monticello, AR. The bark was stored at 4°C until it was ground to 40 mesh using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ) as described by Torget et al. [3].

Extraction:

Two types of extraction methods were tested to remove the value added compounds from the bark. The first type was a solvent extraction, while the second type was a sublimation extraction. For the solvent extraction, two grams of ground bark was combined with 50 ml of distilled water at 85 °C by shaking the mixture for 18 h, in a 200 ml amber bottle, in a Precision shaking water bath (Winchester, VA) at 100 rpm as described by Vaughn et al. [4]. The extract was filtered through #1 Whatman filter paper (Florham Park, NJ) and stored at 4°C until needed. The sublimation extraction was performed by Dr. Philip G. Crandall and Dr. Dinesh Babu, in the Food Science Department at the University of Arkansas. To perform the sublimation extraction, the bark was heated until the compounds began to evaporate after which they were rapidly cooled back to a liquid state. The extract was collected in a 15 ml centrifuge tube and then wrapped in foil to protect it from light and stored at 4°C until further use.

Purification:

The sweetgum bark extract acquired from the sublimation extraction method was purified by centrifugal partition chromatography (CPC). Five ml of the extract were filtered through a 0.45-µm syringe filter (National Scientific, Rockwood, TN) and combined with 5 ml of the solvent system before being injected into the CPC system. The solvent system consisted of ethyl acetate/ ethanol/ water (2:1:2, v/v/v). The solvents were thoroughly mixed and then allowed to separate for at least 2 hours. The ethyl acetate/ethanol rich upper phase was used as the stationary phase, while the water/ethanol rich lower phase was used as the mobile phase.

The CPC separation was performed using a CherryOne DS bench scale CCC control system (Chicago, IL), which utilized an Armen CPC column (Saint-Avé, France), SofTA evaporative light scattering detector (ELSD) (Westminster, CO), and a Foxy R1 Fraction

Collector (Lincoln, NE). The CPC was operated in the descending mode, meaning that the water/ethanol rich phase was the eluent. The 250-mL column was first filled with upper phase at 10 ml/min with the rotor spinning at 500 rpm. Once the rotor was completely filled with the stationary phase, which took approximately 30 min, the CPC rotor speed was increased to 2,500 rpm. At that point, the lower phase was introduced into the CPC rotor at a flow rate of 8 ml/min. The mobile phase volume was determined once the top phase exited the CPC rotor. A 10-ml sample was injected into the 30-ml sample loop and then introduced into the CPC rotor; this was the start of the CPC run. The flow rate was set at 8 ml/min and fractions were collected 20 min after the sample injection, with each sample containing 1 min of eluent, for 120 min, using a Waters Fraction Collector III (Milford, MA).

The eluent was monitored by the ELSD detector with the following setup: 50 psig gas pressure using ultra pure nitrogen, 25°C spray chamber temperature, and 55°C drift tube temperature. From the CPC run, it was determined that the mobile and stationary phases had volumes of 172 and 78 ml, respectively, with the operating pressure at approximately 300 psig. Select fractions along with the crude bark extracts from both extraction methods and a set of standard reference compounds were then analyzed by HPLC.

Analysis:

A Waters 2690 Separation Module High pressure liquid chromatography (HPLC) was used to analyze the bark extracts, CPC fractions, and the set of known standards: ellagic acid, rutin, quercetin, quercetrin, hyperoside, and gallic acid. All samples were filtered with 0.2 µM nylon syringe filters (Nation Scientific, Rockwood, TN) and then injected into a Waters C18 (250mm x4.6mm) column and Waters Symmetry pre-column (Milford, MA) maintained at 30°C. The eluent consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in methanol

(Solvent B). The flow was set at 0.55 ml/min with the initial gradient set at 98:2, Solvent A: Solvent B. The gradient was then linearly decreased to 40% Solvent A over 60 minutes and then returned to 98% Solvent A over 5 minutes. The samples were analyzed with a Waters 996 photodiode array (PDA) detector set at 254 nm.

Antioxidant Activity:

The antioxidant activity of the samples was determined by the TBARS assay as described by Uppugundla et al. 2009 [5]. First, human low density lipoprotein (LDL) was dialyzed in Tris buffer (7.8 g Trizma, 8.7 g NaCl, 1 liter distilled water, 1 N NaOH to adjust pH to 7.4) in Slide-A-Lyzer Mini Dialysis Units from Pierce (10,000 MWCO) at 4°C for 24 hours to remove the ethylenediaminetetraacetic acid (EDTA), which can obstruct the results of the assay. The dialyzed LDL was then combined with Tris buffer to make a LDL solution. For every 2ml of LDL solution needed, 2 ml of Tris buffer was combined with 88 µl of dialyzed LDL. Approximately, 5 ml of the water bark extract, 1 ml of the crude sublimation extract, and 1 ml of select fractions were dried under reduced pressure in an AES 1010 SpeedVac® System (Savant Instruments Inc., Farmingdale, NY) and then reconstituted in 1ml dimethyl sulfoxide (DMSO). The samples were then diluted to produce various concentrations. Standard compounds were also prepared in DMSO at concentrations of 12.5 µM, 50 µM, 100 µM, and 200 µM, while extracts were diluted in terms of 1:1, 1:2, etc.

The assay is essentially carried out as follows. On day one, 10 µl of extract dilutions were added by rows, from less concentrated to more concentrated, to the first four columns of a Microtest^{TM} 96-well, flat-bottom culture plate (Greiner Bio-one, Frickenhausen, Germany). Then 10 µl of freshly prepared 55 µM copper sulfate ($CuSO_4$) and 100 µl of dialyzed LDL solution were added to the initial test wells, immediately followed by the addition of 10 µl of

butylhydroxytoluene (BHT) solution (22 mg BHT, 100 ml ethanol) to the wells of columns 1 and 2 only to stop the oxidation reaction. The plate was then covered with adhesive, air-permeable film (Nalge NUNC International, Rochester, NY) and floated in a water bath (Sheldon Manufacturing, Cornelius, OR) at 37°C for 24 hours.

On day two, 10 µl of BHT solution was added to the wells in columns 3-4 and 60 ml of Tris buffer was added to all initial wells. Calibration standards of triethyl phosphate / tris(hydroxymethyl)aminomethane (TEP/TRIS) were prepared at concentrations of 0 nM, 5 nM, 10 nM, 25 nM and 50 nM as follows. First, 48 µl of TEP was added to 1 liter of Tris buffer to make the TEP/TRIS solution. Next, 0.05 ml of TEP/TRIS solution was combined with 1.95 ml of Tris buffer to make the 5 nM dilution, 0.10 ml of TEP/TRIS solution was combined with 1.90 ml of Tris buffer to make the 10 nM dilution, continuing the pattern until all concentrations were achieved. Exactly 160 µl of each concentration was added, from least concentrated to most concentrated, to the wells corresponding to 10D through 12H in the 96-well flat-bottom plate. Finally, 50 µl of trichloroacetic acid (TCA) solution (30 g TCA, 60 ml distilled water) and 75 µl of thiobarbituric acid (TBA) solution (1.3 g TBA, 7.5 ml 1 N NaOH, 92.5 ml distilled water) were added to all wells, initial and standard. The plate was covered with adhesive film and placed in a water bath at 60 °C for 40 min. The results of the TBARS assay were measured using a Synergy HT microplate reader (BioTek, Winooksi, VT) at 530 nm and 600 nm as described by Uppugundla et al. [5].

On the 96 well flat bottom plates, two replications were performed on each test. For the water extract, two tests were run making the total number of replications (n) equal to 4. The shikimic acid was testing three times, however one of the tests only had one replication, therefore n was equal to 5. The n values of the gallic acid, sublimation extract, and CPC fractions

were 8, 2, and 2 respectively. Error bars were calculated for each concentration using the standard deviation of the replicates.

Results

Extraction:

The extract from the water based extraction was a medium brown color as shown in figure 1. Its brown color was due to a high number of tannins in the bark which are water soluble. As the sweetgum bark chips began to degrade over the year, the color of the extract became more of a dark brown which caused interference with the color based TBARS assay. To solve this problem, a new extract was obtained using the sublimation extraction method. This extract, shown in figure 2, was quite different than the water based extract even though it was made from exactly the same starting material. The extract was a light yellow and appeared oily.

Analysis:

As seen in figure 3, the HPLC analysis of the water based extract showed a few major peaks. In order to try to identify these peaks, a set of standards believed to be in sweetgum bark were run. From this it was determined that the main compound in the water extracted bark was gallic acid, with a retention time of approximately 15.7 minutes. The chromatogram of gallic acid at 0.5 mg/ml is shown in figure 4. The average concentration of gallic acid in the water bark extract was calculated to be 0.049 mg/ml ~ 300uM.

The chromatogram for the sublimation extract, figure 5, showed numerous peaks. The extract was therefore selected for CPC fractionation. After being purified by CPC the array of

compounds were spread across approximately 10 fractions (# 14 - # 24). In figures 6 – 8, the separation of the compounds is shown for fractions #15, #19, and #23 respectively.

Purification:

The chromatogram, shown in figure 9, shows compound detection during the CPC run. The red line indicates detection at 254nm while the yellow line indicates ELSD detection. The fractions were collected starting at 48 minutes, with majority of the compounds being detected between 55 and 90 minutes.

Antioxidant Activity:

First, the crude water bark extract was tested for antioxidant activity. As shown in figure 10, the extract began to display activity at a concentration of 12.5mg bark/ ml water. Next, the antioxidant activity of pure reference compound shikimic acid (figure 11) was tested for antioxidant activity. Unfortunately, it did not display antioxidant activity even at strong concentration, so it was ruled out as a contributor to the bark's activity. Through HPLC analysis, it was determined that the crude bark extract contained gallic acid, which was shown to be a strong antioxidant with activity starting at just 50µM (figure 12).

The sublimation extract, already being a liquid, was diluted with DMSO and tested for antioxidant activity. The extract was an extremely potent antioxidant with activity starting at 0.78% extract in DMSO as shown in figure 13. After fractionation by CPC, fractions 7, 11, 15, 19, 23, 27, and 31 were tested (figure 14). Fractions 15, 19, and 23 displayed strong antioxidant activity while the rest did not.

Conclusion and Discussion

The sublimation extraction method removed more compounds and at stronger concentrations. The water extraction method also removed value added compounds, such as gallic acid. The water extraction method has been proven to increase dilute acid pretreatment results and is therefore very applicable to the biofuels industry once it has been scaled up. The sublimation extraction method leaves the bark very dry and could therefore be implemented into the paper industry, which usually combusts the bark to extract its thermal energy.

The HPLC analysis of the water extracted sweetgum bark indicates the presence of gallic acid in the bark. The bark extract, as well as gallic acid, were shown to be strong antioxidants. This suggests that gallic acid may be one of the primary contributors to the antioxidant activity of sweetgum bark. The complexity of the sublimation extract will require mass spectrometry to determine which compounds are present in the crude extract and in the corresponding fractions.

Although more testing will need to be done to identify all of the compounds present in the bark and their antioxidant activity, it is evident that the bark of sweetgum trees does contain valuable chemicals such as gallic acid that can be removed with a water treatment prior to the conversion into biofuels.

Figures

Figure 1: Water-based bark extract

Figure 2: Sublimation bark extract

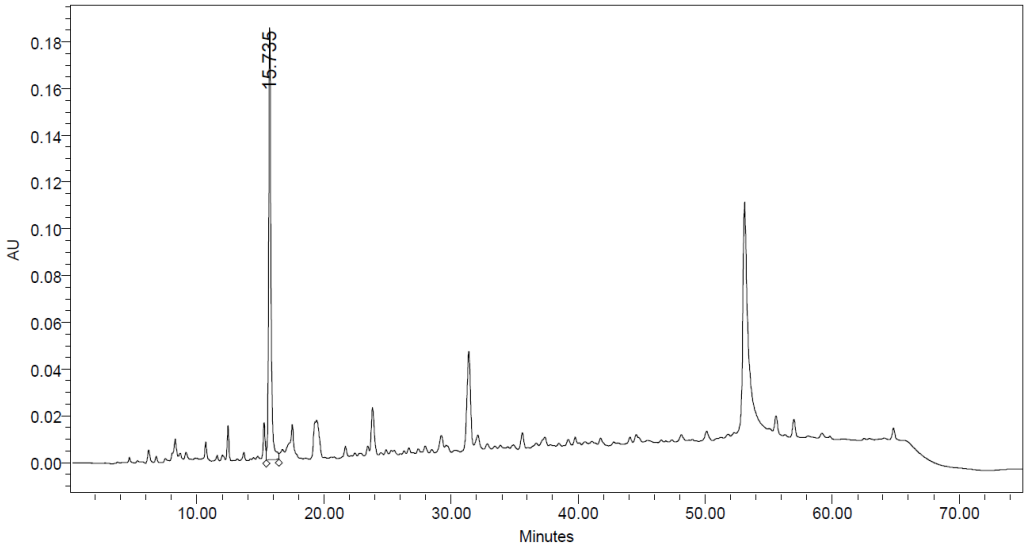

Figure 3: HPLC chromatogram of the water-based bark extract

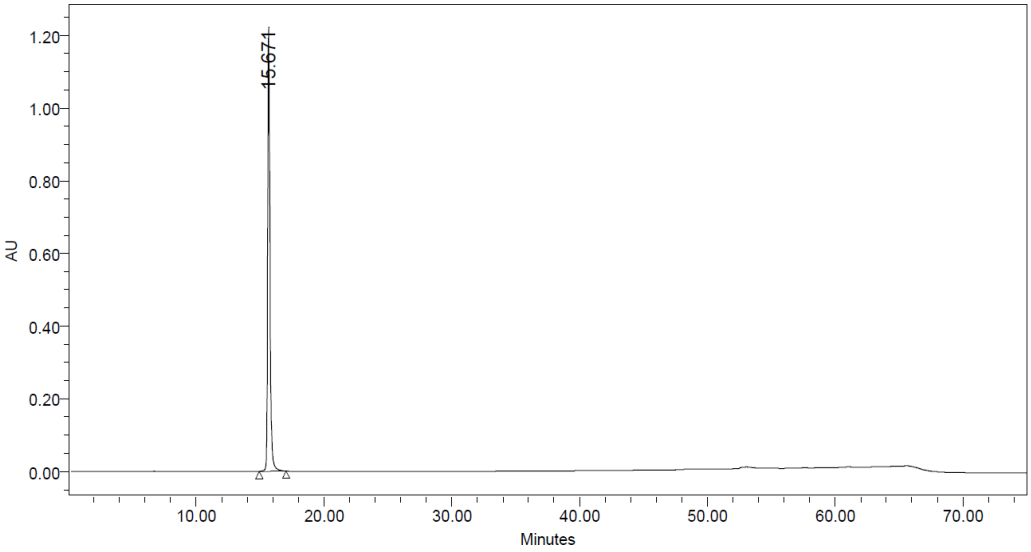

Figure 4: HPLC chromatogram of the gallic acid standard at 0.5mg/ml

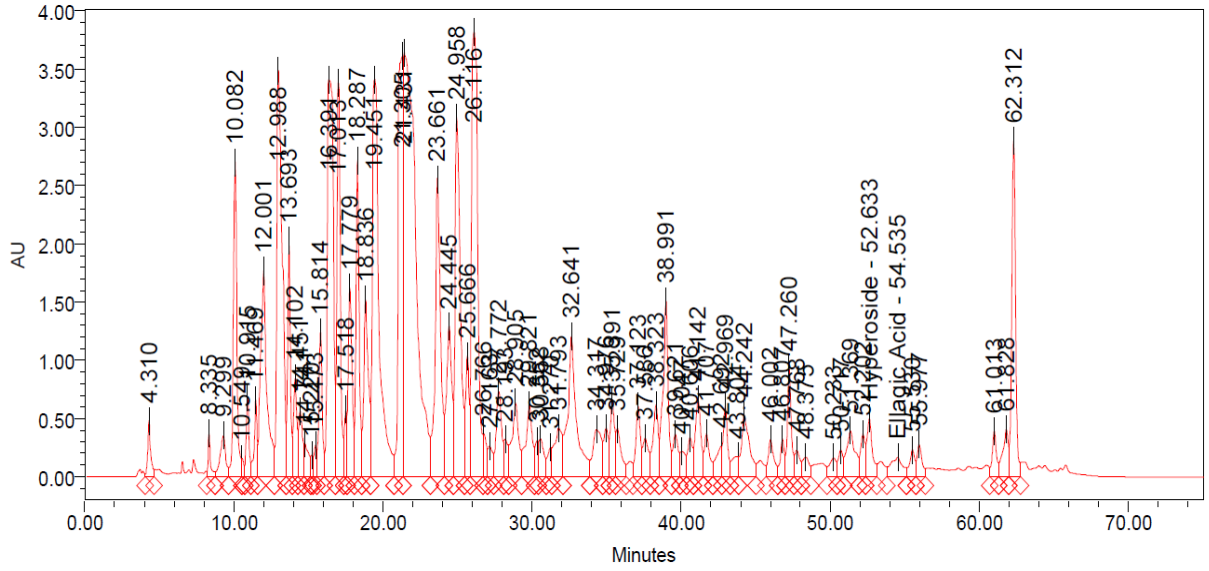

Figure 5: HPLC chromatogram of the sublimation bark extract

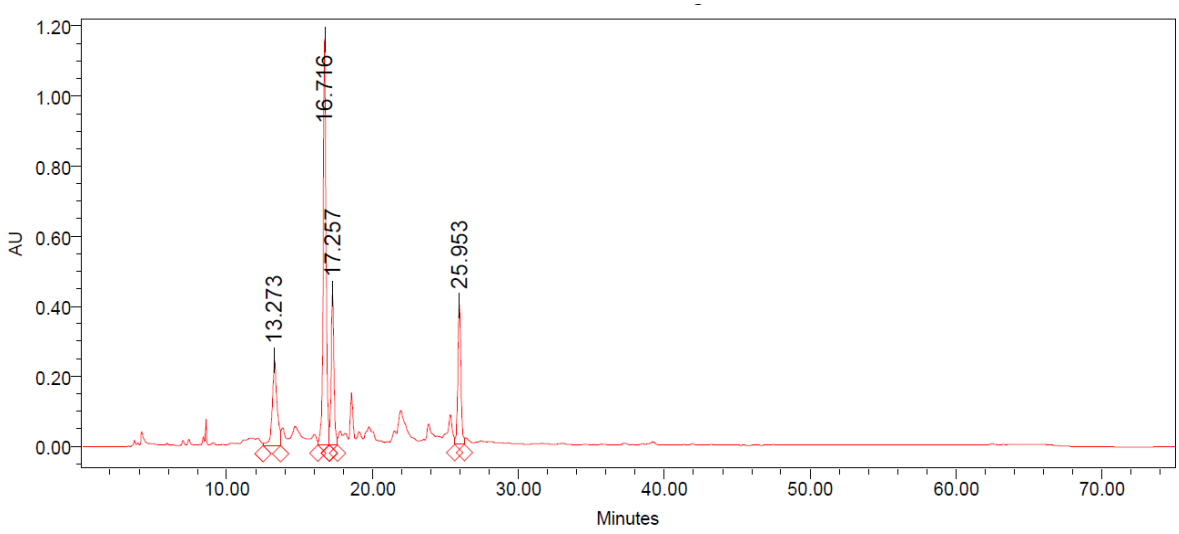

Figure 6: HPLC chromatogram of fraction #15

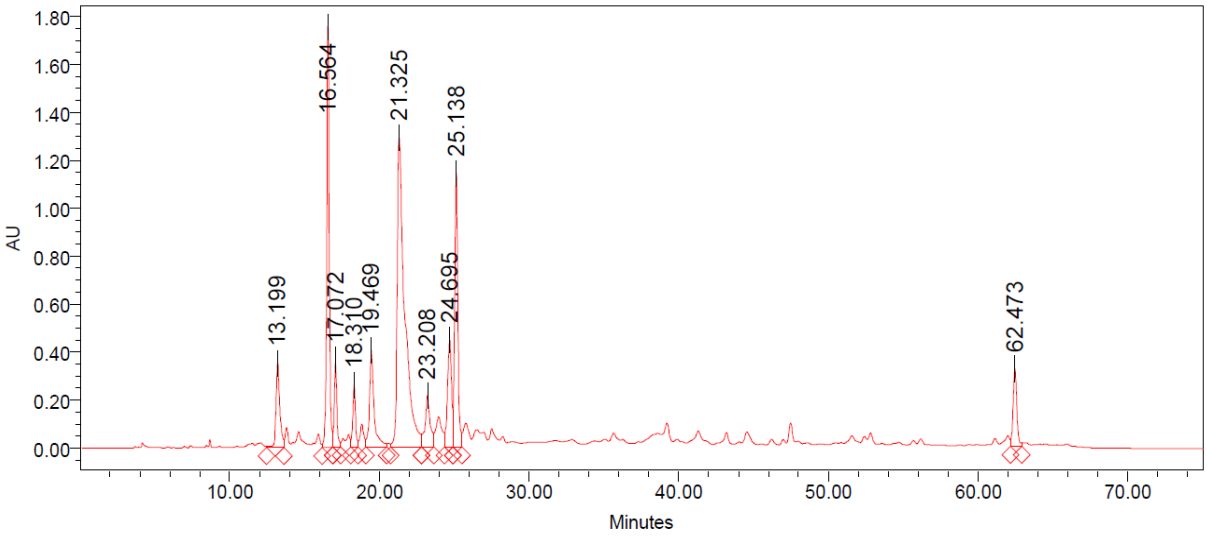

Figure 7: HPLC chromatogram of fraction #19

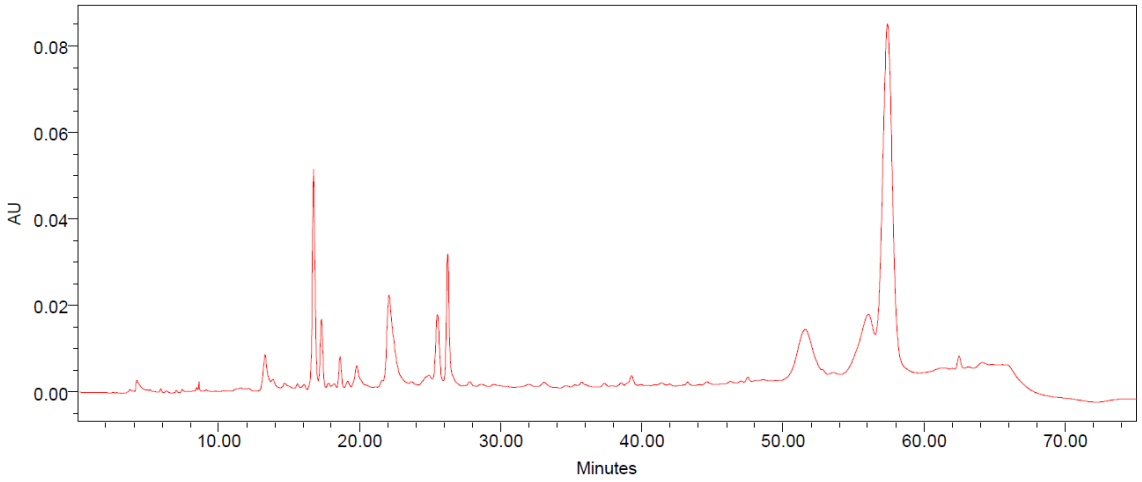

Figure 8: HPLC chromatogram of fraction #23

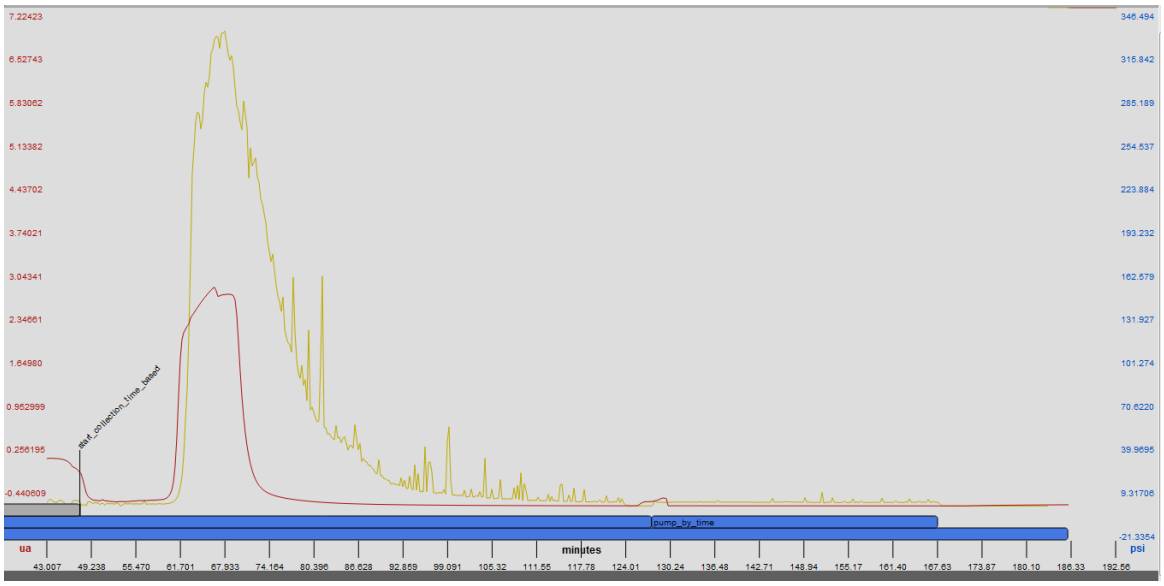

Figure 9: Chromatogram of CPC run

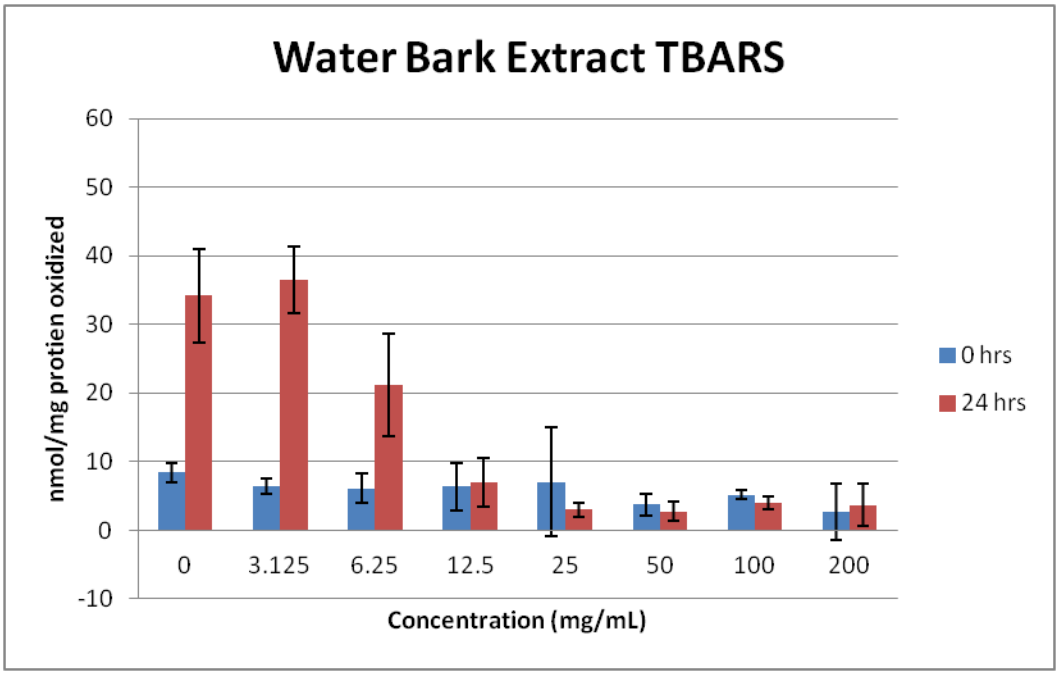

Figure 10: Water extracted bark TBARS graph (n = 4)

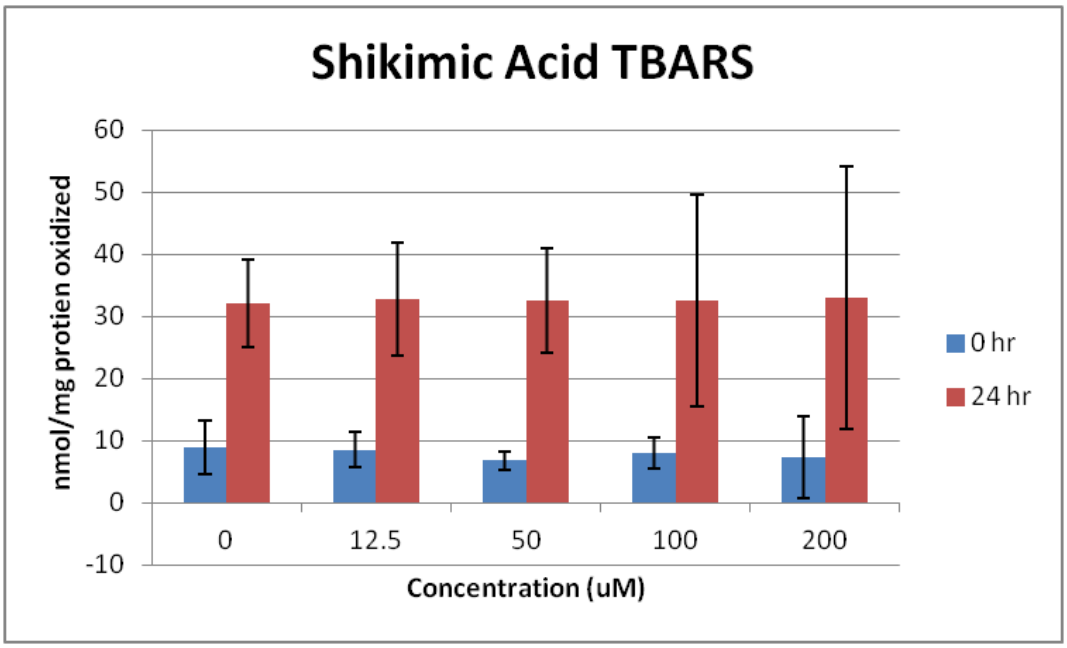

Figure 11: Shikimic acid TBARS graph (n = 5)

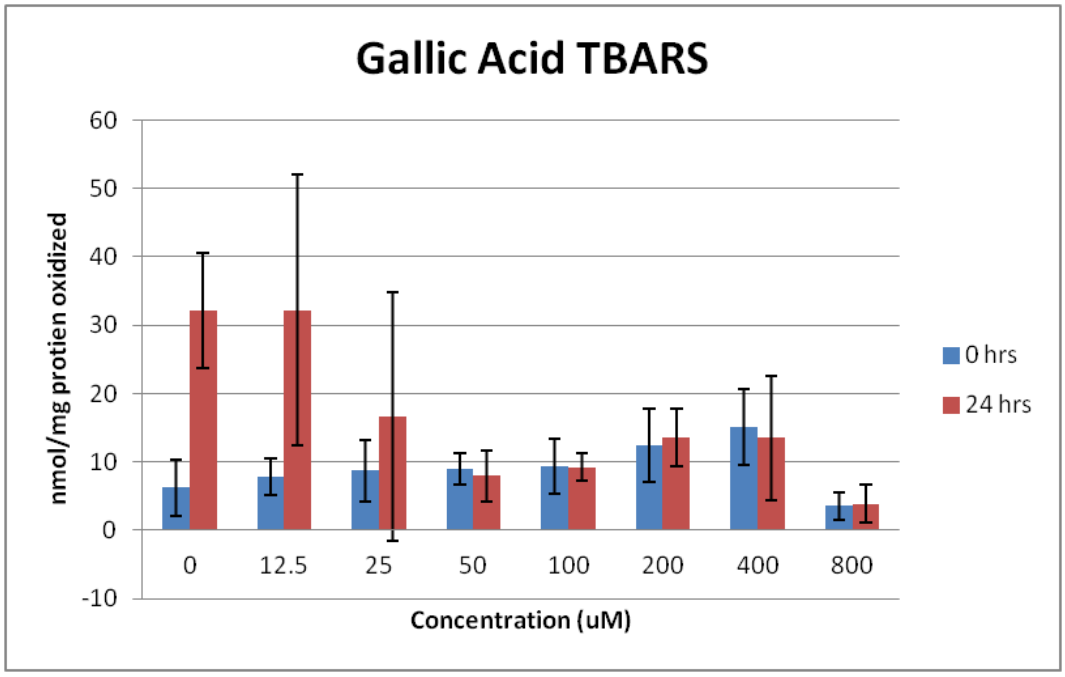

Figure 12: Gallic acid TBARS graph (n = 8)

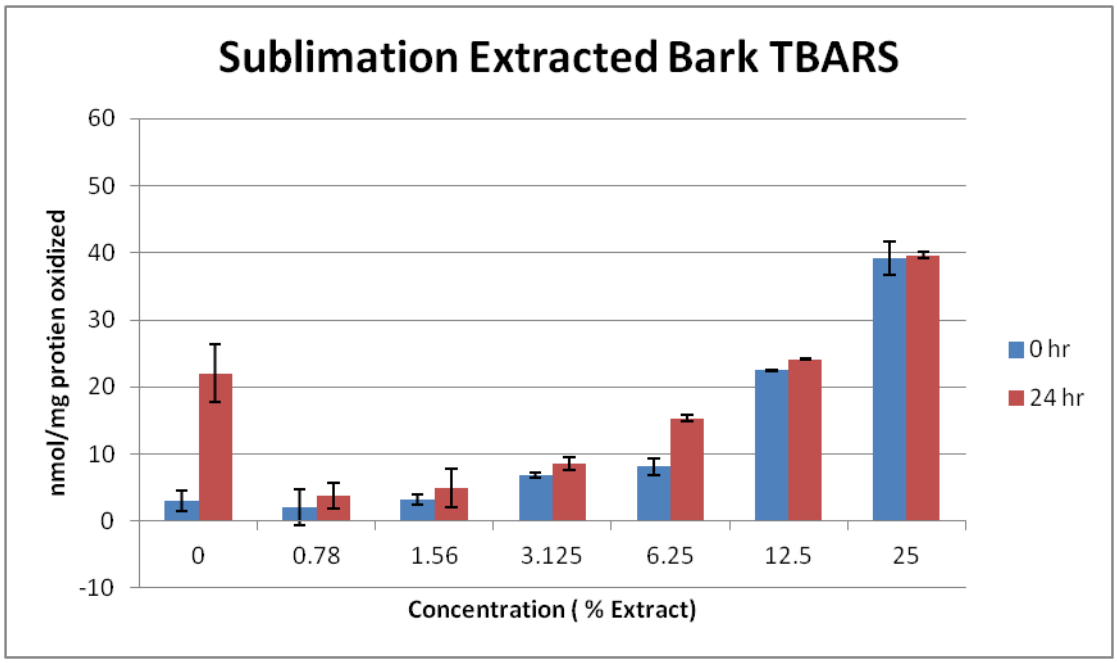

Figure 13: Sublimation extracted bark TBARS graph (n = 2)

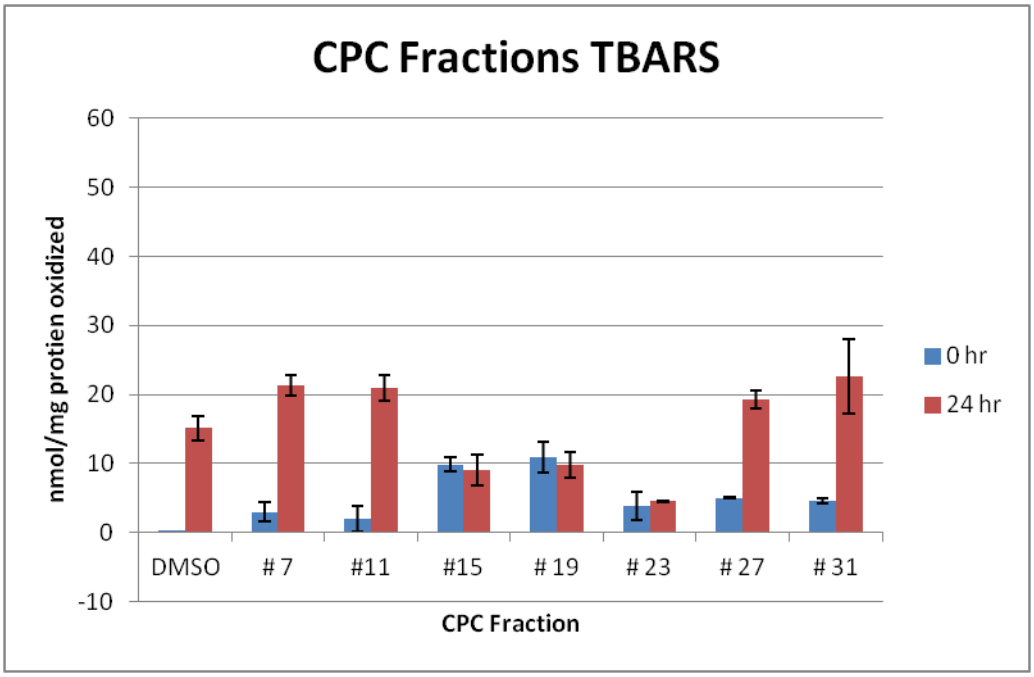

Figure 14: CPC fractions TBARS graph (n = 2)

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